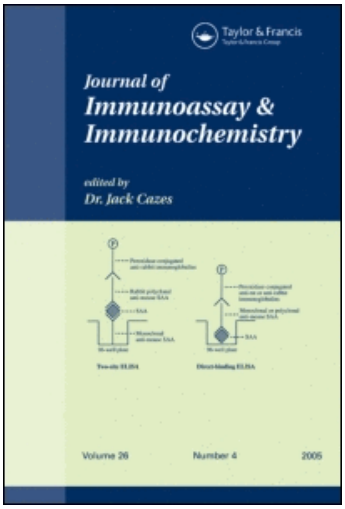

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ABSTRACT

Tuberculosis (TB) has re-emerged as a major health problem worldwide. Developing an easy, inexpensive immunodiagnostic test is extremely important for TB diagnosis, especially in developing countries. A target mycobacterial circulating antigen of 55-kDa molecular weight was identified in sera from confirmed *Mycobacterium tuberculosis* infected individuals by using Western blotting based on a specific mouse IgG anti-*M. tuberculosis*

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monoclonal antibody (TB-55 mAb). No bands were identified in sera of healthy individuals. The target TB antigen was isolated and characterized as a protein. It consists of 15 amino acids; 24.6% of the amino acids are hydrophobic and 46.4% are hydrophilic. A dot-ELISA format, based on TB-55 mAb, was developed for the direct demonstration of the 55-kDa TB antigen in serum samples of pulmonary TB patients. The technical aspects of the developed dot-ELISA are simple, rapid (5 min), and reproducible, as well as sensitive (87%) and specific (93%). Using the more sensitive immunoassay; Western blot, the 55-kDa TB antigen was detected in all (100%) sera that have been shown false negative by dot-ELISA, as well as in true positive sera. In conclusion, we have developed a simple and rapid immunoassay for the direct detection of a circulating mycobacterial antigen in sera of TB infected individuals and, therefore, the developed assay can be applied for laboratory and field diagnosis of TB infection in developing countries.

INTRODUCTION

Recent estimates by The World Health Organization (WHO) suggest that a third of the world's population is infected with *Mycobacterium tuberculosis* and about 8 million individuals developed clinical tuberculosis (TB) last year.[1] The mainstay for its control is the rapid and accurate identification of infected individuals. At present, TB diagnosis in developing countries relies largely on clinical features, X ray, and the simple and rapid detection of acid-fast bacteria (AFB) in sputum by direct microscopy using Ziehl-Neelsen staining. In practice, around 40 to 60%, at best, of all adults with pulmonary TB can be identified with the current direct microscopy test.[2] Culture of bacteria as a diagnostic "gold standard" for tuberculosis is a costly (and, therefore, not ubiquitously available) and complex technique, requiring up to several weeks before a definite diagnosis is established.[3]

The tuberculin skin test is not useful in subjects with a previous history of active tuberculosis or *Mycobacterium bovis* BCG vaccination. Polymerase chain reaction (PCR) has been successfully introduced for rapid diagnosis of TB and has the capability to distinguish between different species of *Mycobacterium*. However, about 50% sensitivity of PCR was reported among acid-fast bacillus smear-negative pulmonary tuberculosis.[4,5] In addition, PCR is expensive and relies on sophisticated equipment, where these conditions are often lacking in developing countries.

There has been strong demand for the development of rapid, reliable, and less costly immunodiagnostic methods for the detection of pulmonary TB.[3,6] Numerous immunological methods that use various modifications of ELISA or immunochromatographic methods have been developed for the detection of specific antibody classes to various TB antigens.[7–9] However, people in developing countries, particularly in tropical areas, are in contact with various pathogens and developed cross-reacting antibodies responsible for poor specificity.[10] In addition, the sensitivity of antibody tests is much lower in HIV seropositive patients coinfected with tuberculosis.[11] Recently, more efforts are directed toward developing rapid, reliable, and less costly immunoassays based on the detection of TB antigens in body fluids using specific monoclonal antibodies. Such tests could be useful for the diagnosis and follow-up of patients.[12]

In the present study, we have developed an IgG monoclonal antibody, designated TB-55 mAb, and the TB circulating target antigen was isolated and characterized. The sensitivity and specificity of serum antigen detection with a developed dot-ELISA format based on TB-55 mAb were investigated for rapid and simple diagnosis of pulmonary tuberculosis.

EXPERIMENTAL

Serum Samples

Serum samples of 167 patients (100 males and 67 females, aged 14–58 yr) were obtained from the respiratory clinic of El-Abasia Chest Hospital, Cairo, Egypt. They were diagnosed as having active pulmonary tuberculosis by acid-fast bacilli using Ziehl-Neelsen staining and culture of sputum, in combination with confirmed clinical symptoms. Only 7 sera were of patients showing negative smear and positive culture results. The blood and sputum samples were obtained from all patients before initiation of treatment. In addition, serum samples of another 28 patients (17 males and 11 females, aged 15–58 yr) were included. They were diagnosed as having respiratory diseases other than pulmonary tuberculosis, based on their negative sputum smears in combination with radiographs and clinical symptoms. These respiratory diseases included asthma, pneumonia, bronchitis, lung cancer, and lung infection. Blood samples were collected from 55 healthy volunteers (33 males and 22 females, aged 15–56 yr) as a control group. Blood was allowed to clot

at room temperature; serum was separated by centrifugation at 3000 rpm for 5 min, then an aliquot was taken and stored at −70°C until tested. All individuals who participated in the present study showed no detected antibodies to HIV and were fully informed concerning the diagnostic procedures involved and the nature of the disease.

Monoclonal Antibody (TB-55 mAb)

An IgG anti-*M. tuberculosis* mouse monoclonal antibody, designated TB-55 mAb, was prepared using a hybridoma technique as follows. *M. tuberculosis* was grown at 37°C for 4–6 weeks on Lowenstein-Jensen medium. Total bacterial culture filtrate was collected by filtration through 0.45 μm cellulose acetate membrane, and then dialysed at 4°C against 0.01 M PBS, pH 7.2, for 24 h. The dialyzed filtrate was stored at −70°C for 60 min, lyophilized, and reconstituted with 0.01 M PBS, pH 7.2. Protein content was determined and it was stored at −20°C. Balb/c female mice were intraperitonealy immunized using the dialyzed bacterial culture filtrate. Spleen cells were taken from immunized mice and fused with P3-X63-Ag8-UI mouse myeloma cells. The resulting hybrids were tested for the presence of specific antibodies against *M. tuberculosis* cultural filtrate using an indirect enzyme linked immunosorbent assay (ELISA).

The highly positive hybrids were cloned by the limiting dilution method. One of the highly reactive cell lines (designated TB-55) indicating the specificity of the developed TB-55 mAb to *M. tuberculosis*, was injected intraperitonealy into Balb/c mice for ascites production. The ascites were collected, centrifuged to remove the debris, and stored at −20°C until used.

SDS-PAGE and Gel Electroelution

Serum samples were subjected to SDS-PAGE, at 30 μg/lane, using vertical slabs of 12% or 16% polyacrylamide.[13] Molecular-weight standards (Promega, Madison, WI) were run in parallel. After silver staining,[14] the band of interest (55-kDa) was cut and the antigen electroeluted from the polyacrylamide[15] at 200 V for 3 h in a dialysis bag (Sigma). After dialysis, the electroeluted antigen was concentrated using polyethylene glycol and 40% trichloroacetic acid (TCA), then centrifuged at 6500 *g* for 15 min. The precipitate was washed twice using

diethyl ether to remove the excess of TCA. The excess diethyl ether was removed by gentle drying and the pellet was reconstituted in PBS, pH 7.2. The protein content of a sample of the electroeluted antigen was determined before the remainder was stored at −20°C until used.

Western Blots

Samples separated on SDS-PAGE and were electrotransferred onto a nitrocellulose (NC) membrane (0.45 μm pore size, Sigma) in a protein transfer unit.[16] The NC filter was blocked using 5% (w/v) non-fat dry milk dissolved in 0.05 M Tris-buffered saline (TBS), containing 200 mM NaCl (pH 7.4), rinsed in TBS, and incubated with TB-55 mAb diluted in blocking buffer with constant shaking. The blots were washed 3 times (30 min each) in TBS, followed by incubation for 2 h with anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted 1:500 in TBS. After washing 3 more times with TBS (15 min each), the blots were soaked in substrate (premixed 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) in 0.1 M Tris buffer, pH 9.6; ABC Diagnostics, New Damietta, Egypt). The color reaction was observed within 10 min, and then dipping the blots in distilled water stopped the reaction.

Dot-Enzyme Linked Immunosorbent Assay (Dot-ELISA)

We have developed a simple and rapid dot-ELISA format to detect the target circulating antigen of TB-55 mAb. All the following assay steps were carried out on the surface of a nitrocellulose (NC) membrane fixed in a plastic cartridge device (see Fig. 2) and each reagent was completely absorbed into the NC membrane within 30 s (incubation time). After optimization of assay conditions and characteristics, the following procedure was used. Two hundred micro-liters of serum sample diluted 1:20 in 50 mM EDTA, were added per dot. Different concentrations of the purified TB antigen were used as positive controls and sera from non-infected healthy individuals were used as negative controls. Blocking of the non-specific binding sites on the NC filter was done with 5% (w/v) BSA in PBS, pH 7.2. TB-55 mAb was then added in a dilution of 1:300 in PBS. After washing, the anti-mouse IgG alkaline phosphatase conjugate (Sigma) was added. After more washing, the premixed NBT/BCIP substrate in 0.1 M Tris buffer (ABC Diagnostics) was applied and left on the dot for 2 min before

the reaction was stopped by adding 100 μL distilled H_2O and the color development was observed. The resultant color was tested and read by the naked eye and a single individual without knowledge of subject status. The development of a violet color indicates antigen detection. The color of tested serum sample was compared and related to one of the positive control levels.

Characteristics of the Purified Antigen

Purity

The purity of the isolated 55-kDa TB antigen was assessed using capillary zone electrophoresis (CZE) in a modification of the method described by Gordon et al.,[17] using an autosampler (Model 1-LIFT; Prince Technologies, Emmen, The Netherlands), a 65 cm fused silica capillary (75 μm inner diameter) coated with polyimide film (Prince Technologies), a variable ultra-violet–visible detector (Lambda 1010; Metrohm, Herisau, Switzerland), and WinPrince software (version 5; Prince Technologies). For the CZE run, 10 μL of a dilution of the purified antigen (50 μg/mL distilled water) were injected through the capillary at high voltage (30 kV) and low pressure (25 mbar) for 10 s before the sample was eluted with borate buffer (pH 8.3) at high voltage (30 kV) for 15 min while the internal capillary temperature was kept at 20°C. Eluents were detected by their ultra violet absorption at 200 nm and the signals were analyzed using Dax software (version 5; Prince Technologies).

Chemical Type

To determine some of the antigen chemical characteristics, samples of the antigen were treated with protease and one of several other chemical reagents being tested in the dot-ELISA, to see if these treatments affected the active epitope. The purified antigen, at 1 mg/mL, was incubated for 1 h, either with 40% TCA (v/v) at 4°C or with 0.2 M NaOH or 0.2 M HCl (v/v) at room temperature (RT). A periodate oxidation was carried out overnight with 20 mM sodium *meta*-periodate at RT and the reaction was then inhibited by adding an equal volume of 130 mM glycerol. In another series of experiments, a sample of the purified antigen (at 200 μg/mL) was mixed with an equal volume of 180 mM β-mercaptoethanol. In the test with protease, purified antigen (1 mg/mL) was incubated at 37°C with

protease of type VIIA from *Bacillus amyloliquefaciens* (1 mg/mL; Sigma) for 1 h or with α-chymotrypsin (1 mg/mL; Sigma) for 45 min. In the dot-ELISA, purified antigen and BSA were tested in parallel, as positive and negative controls, respectively.

Amino Acid Content

One-mg of the purified antigen was hydrolyzed, under vacuum, for 24 h at 120°C, in 6 M HCl containing 1% phenol. The hydrolyzed sample was dried and derivatized with phenyl isothiocyanate. A mixture of 18 amino acids was prepared in 0.1 N HCl and used for calibration. The derivatized amino acids were then analyzed using reversed-phase HPLC in a 25-cm Spherisorb C_8 column (4.6 mm inner diameter; Kontron, Zurich, Switzerland) using a personal computer integration pack (Kontron). The injection volume was 10 μL. The mobile phase employed for the separation consisted of two eluents: A (0.1 M sodium acetate containing EDTA; pH 5.5); and B (an organic phase consisting of $CH_3CN/CH_3OH/H_2O$ at 45:40:15, by volume. The flow rate was 1.5 mL/min and the detection was performed at 254 nm using a gradient varying from 6% eluent B to 45% eluent B in 60 min.

RESULTS

Identification of the Target *M. tuberculosis* Circulating Antigen of TB-55 Monoclonal Antibody

The target antigen of the TB-55 mAb was identified by the Western blot at 55-kDa in serum samples of active TB infected individuals, as shown in Fig. 1. No bands were identified in sera from non-infected controls.

Biochemical Characterization of the Target TB Antigen

The 55-kDa-target antigen was purified from sera of active TB infected patients using electroelution from polyacrylamide preparative slab gels. The purified antigen showed a single peak when analyzed by capillary zone electrophoresis (data not shown). The reactive epitope was sensitive (i.e., showed no reactivity using dot-ELISA) to acid and base hydrolysis, β-mercaptoethanol, protease, and pepsin treatments.

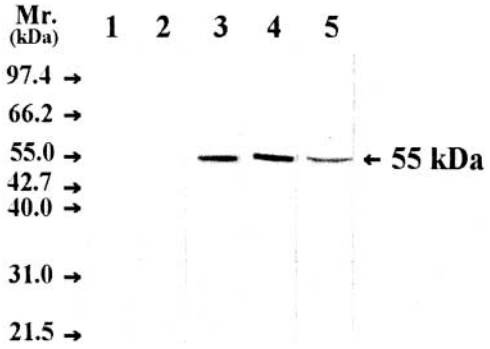

Figure 1. Western blot based on TB-55 mAb of serum samples from *M. tuberculosis* infected patients and from healthy individuals. Lanes 1 & 2: sera of 2 confirmed non-infected healthy individuals, lanes 3–5: sera from 3 confirmed *M. tuberculosis* infected patients. Samples were separated on 12% polyacrylamide gels in presence of SDS and resolved proteins were electroblotted onto NC membrane and probed with TB-55 mAb. All infected patients showed the target 55-kDa antigen. Molecular weight (Mr.) markers were not shown and indicated by arrows.

The antigen was precipitated with 40% TCA, and reconstituted in PBS, pH 7.2. The reconstituted precipitate showed high reactivity (i.e., colored dot) toward the TB-55 mAb. In contrast, the supernatant showed no reactivity (colorless dot). Periodate treatment did not affect the reactivity of the target epitope. Amino acid compositional analysis revealed that the 55-kDa antigen contains 24.6% hydrophobic amino acids, of which 46.4% were hydrophilic, 12.7% acidic amino acids, and 16.3% basic amino acids (Table 1).

Rapid Detection of TB Circulating Antigen in Sera Using Dot-ELISA

We have developed a simple dot-ELISA format, based on TB-55 mAb, for rapid detection (within 5 min) of 55-kDa TB antigen in serum samples of patients infected with pulmonary TB. The dot-ELISA, based on TB-55 mAb, allows a semi-quantitative reading of the resulting colored spot in case of TB antigen detection (i.e., positive test). The developed purple color varied in its intensity, from faint (1+ or 2+) to dark (3+ or 4+). Colorless dot (negative test) was produced

Table 1. Amino acid concentrations of the 55-kDa *M. tuberculosis* antigen.

Groups of amino acids	Amino acids (abbreviation)	Concentration (nmol/mg protein)	Relative percentage	Total percentage
Hydrophobic	Leucine (Leu)	47.14	2.1	24.6
	Isoleucine (Ile)	49.28	2.2	
	Valine (Val)	81.42	3.7	
	Proline (Pro)	59.28	2.7	
	Methionine (Met)	98	4.4	
	Tyrosine (Tyr)	94.28	4.3	
	Alanine (Ala)	113.14	5.2	
Hydrophilic	Glycine (Gly)	755.71	34.2	46.4
	Serine (Ser)	214.28	9.7	
	Theronine (Thr)	55.71	2.5	
Basic	Lysine (Lys)	162.85	7.4	16.3
	Arginine (Arg)	85.71	3.9	
	Histidine (His)	111.42	5.0	
Acidic	Glutamic acid (Glu)	135.71	6.2	12.7
	Aspartic acid (Asp)	142.85	6.5	

in case of no antigen detection, i.e., negative test, (Fig. 2). Sera of 195 patients from the respiratory clinic and 55 healthy controls were tested using dot-ELISA. The target TB circulating antigen was detected using dot-ELISA in 145 (87%) of 167 serum samples of patients infected with pulmonary TB. The assay detected the TB target antigen in 6 (86%) out of 7 sera from individuals showing negative smear and positive culture results. The 22 false negative TB samples were tested using the more sensitive Western blot and the 55-kDa antigen was detected in all (100%) false negative samples, as well as in all true positive samples. The dot-ELISA assay showed 2 false positive results among 28 patients with the respiratory diseases other than tuberculosis and 4 false positive results among 55 healthy controls; this revealed an overall specificity of 93%.

DISCUSSION

The current resurgence of tuberculosis (TB) worldwide, and particularly in developing countries, has created a need for less expensive

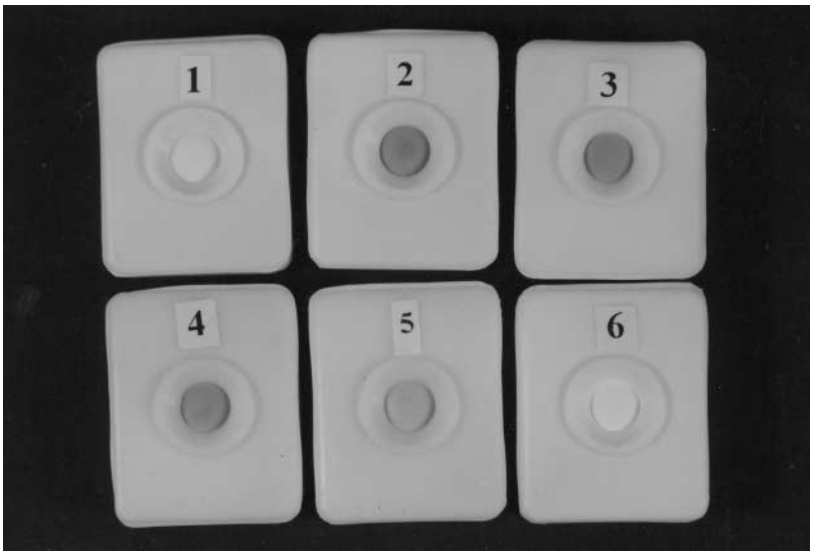

Figure 2. Detection of TB antigen in serum samples of *M. tuberculosis* infected individuals and non-infected individuals using dot-ELISA. The device labeled number 1 represents negative control and devices 2 and 3 represent positive controls (100 and 50 μg per dot of purified 55-kDa TB antigen, respectively). Devices 4 and 5 represent serum samples from two *M. tuberculosis* infected individuals (one showing high antigen level [4+] and the other one showing low antigen level [1+] and device 6 represents serum from non infected individual showing no detection of antigen, respectively).

and more effective diagnostic techniques.[6] Preliminary results have been published on new diagnostic tests for tuberculosis, based on detection of mycobacterial antigens in different body fluids. The development of anti-*M. tuberculosis* mAbs had led to identification of several antigens with somewhat different characteristics and molecular masses.[18–20] Espitia et al.[21] identified and characterized *M. tuberculosis* 50- to 55-kDa antigen as a glycoprotein, as assessed by binding of concanavalin A, labeling of carbohydrate moieties with biotin-hydrazide, and digestion of carbohydrates with jack bean alpha-D-mannosidase. Total amino acid analysis of the major 55-kDa component of *M. tuberculosis* 50- to 55-kDa glycoprotein showed that proline and alanine were the predominant amino acids, i.e., it is a proline-rich mycobacterial protein. In the present study, TB-55 mAb, developed against *M. tuberculosis* cultural filtrate, recognized a 55-kDa antigen in sera of active TB infected individuals.

The protein nature of the reactive epitope was confirmed by several experiments. Periodate oxidation did not affect the reactivity of the antigen. Total amino acid analysis revealed high percentages of hydrophilic amino acids; this might contribute to immunogenicity and stability of the 55-kDa reactive epitope. Further analytical work and results of current work will help to establish the structural basis for the common features. Mycobacterial antigens have been detected by ELISA in sputum,[12,19,22] serum,[23,24] cerebrospinal fluid,[25] and by latex agglutination assay or dot-immunobinding assay (Dot-Iba) in cerebrospinal fluid of patients with tuberculosis.[26,27] However, none of these tests to detect mycobacterial antigens has become available for clinical use nor achieved widespread use for the diagnosis of active tuberculosis. In the present study, a dot-ELISA, based on IgG TB-55 mAb, was developed for simple detection of 55-kDa antigen in serum within 5 min. The developed dot-ELISA could be useful for laboratories with limited resources and limited technical experience. The developed assay showed high sensitivity (87%) and specificity (93%). In addition, the assay detected the TB target antigen in 86% of sera from individuals showing negative smear and positive culture results.

The false negative results of the developed dot-ELISA may be explained as follows. The TB target antigen level among false negative samples may be too low to be detected. The target 55-kDa antigen was identified by using the more sensitive Western blotting in all (100%) false negative TB samples. In addition, mycobacterial antigens have been found as components of circulating immune complexes,[30] so it may be necessary to dissociate the immune complexes to achieve a higher sensitivity in the immunoassay.[12,26,29,30] In antigen detection assays, sample processing is often too laborious for daily use in laboratories in endemic areas and involves time consuming steps. However, the serum samples will be pretreated in detergents to increase the developed assay sensitivity. This pretreatment inactivates the antibodies and simultaneously, the antigens are released, and the epitopes are exposed.

The clinical diagnosis of pulmonary TB is often problematic. A number of respiratory diseases such as pneumonia, bronchitis, and cancer can mimic both clinical symptoms and the shadow often seen on a radiograph with pulmonary TB patients.[31] Most patients with respiratory diseases other than TB showed negative results using dot-ELISA, although 7% were positive. As all patients with respiratory disease other than TB were sputum negative, only radiographs and clinical symptoms were used for their diagnosis. Therefore, it is possible that concurrent TB infection could also be present in some patients with

respiratory disease other than TB, diagnosed with circulating antigen detection, i.e., showing false positives.

In conclusion, we have developed a simple and rapid dot-ELISA format for the detection of circulating TB antigen in serum. The developed assay could be used as a rapid screening tool for TB in the developing countries. Further studies regarding the detection of the target TB antigen, before and after treatment of TB infected patients, will be performed.

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